

THE EFFECT OF SIMULATED MICROGRAVITY ENVIRONMENT OF RWV BIOREACTORS ON SURFACE REACTIONS AND ADSORPTION OF SERUM PROTEINS ON BONE-BIOACTIVE MICROCARRIERS

S. Radin^{1*}, P. Ducheyne^{1**}, and P. S. Ayyaswamy²

Center for Bioactive Materials and Tissue Engineering,

¹Department of Bioengineering, ²Department of Mechanical Engineering and Applied Mechanics, School of Engineering and Applied Sciences, University of Pennsylvania, 220 S 33rd St., Philadelphia, PA

Abstract

Biomimetically modified bioactive materials with bone-like surface properties are attractive candidates for use as microcarriers for 3-D bone-like tissue engineering under simulated microgravity conditions of NASA-designed rotating wall vessel (RWV) bioreactors. The simulated microgravity environment is attainable under suitable parametric conditions of the RWV bioreactors. Ca-P containing bioactive glass (BG), whose stimulatory effect on bone cell function had been previously demonstrated, was used in the present study. BG surface modification via reactions in solution, resulting formation of bone-like minerals at the surface and adsorption of serum proteins is critical for obtaining the stimulatory effect. In this paper, we report on the major effects of simulated microgravity conditions of the RWV on the BG reactions surface reactions and protein adsorption in physiological solutions. Control tests at normal gravity were conducted at static and dynamic conditions. The study revealed that simulated microgravity remarkably enhanced reactions involved in the BG surface modification, including BG dissolution, formation of bone-like minerals at the surface and adsorption of serum proteins. Simultaneously, numerical models were developed to simulate the mass transport of chemical species to and from the BG surface under normal gravity and simulated microgravity conditions. The numerical results showed an excellent agreement with the experimental data at both testing conditions.^{1,2}

Introduction

Studies from space flights over the last two decades have demonstrated that there are basic physiological changes in humans during space flight, including severe loss of calcium and mineralized bone.³ Microgravity has been noted to modify the function of the bone cells and disturb metabolism.⁴ With the planned long duration space travel and stay, there is a great need to gain a fundamental understanding of the effect of microgravity on human bone cell function. Several aspects of microgravity conditions of outer space can be simulated on earth using NASA designed Rotating Wall Vessel (RWV) bioreactors.⁵ In the RWVs, cells may be seeded on suitable microcarriers and their function determined in comparison to the function under normal gravity conditions. An important aspect is the development of a “suitable” microcarrier to successfully enable the study of this problem in simulated microgravity. In this study we hypothesize that studies focusing on understanding bone loss are conducted in a near optimal way if the microcarriers have properties akin to those of the bone mineral phase.

Keywords: simulated microgravity, microcarriers, protein adsorption, surface reactions, 3D tissue engineering, biomaterials

* Corresponding author. e-mail: radin@seas.upenn.edu ** Principal Investigator

Ca-P containing bioactive glass (BG) as a material, which forms bone-like minerals upon immersion in physiological solutions, is a suitable microcarrier material for the fundamental studies of microgravity on bone cell function. As was demonstrated in our previous studies, surface modified BG produces a major effect on bone cells; namely, osteoprogenitor cells are upregulated to osteoblasts.⁶ This effect on cells is obtained by modifying the glass surface in solution.⁶ This treatment results in surface reactions and in the formation of a Ca-P layer at the BG surface. In solution, this surface can further transform to a bone-like mineral, i.e., carbonated crystalline hydroxyapatite (c-HA)⁷ We have also demonstrated that this transformation is dependent on the composition of the milieu in which the glass is immersed including the presence or not of proteins.^{8,9} It further followed from our work that there is a selective adsorption of attachment molecules (fibronectin) without conformational changes that would affect their ligand function.^{6,10} As a material with the unique, bone-like surface properties, this surface modified bioactive glass is an attractive candidate for use as microcarrier material for 3-D growth of bone-like tissue under simulated microgravity conditions of the RWV bioreactors. Considering that the RWV environment is completely different from conditions previously used for BG testing at normal gravity, determination of the BG surface reactions in the RWV was required.

In this paper, we report on the effect of simulated microgravity on the BG surface transformation reactions. We used the NASA designed High Aspect Ratio Vessel (HARV) for simulating the microgravity conditions. We describe the choice of experimental parameters, which ensured simulated microgravity conditions of BG particles in the HARV, the immersion experiments, and the analysis of BG reactions. We also describe the results of a numerical study of the transport of momentum and species in the HARV for the experimental conditions employed in this paper.

Materials and Methods

Bioactive glass 45S5⁷ (Mass %: 45.0 % SiO₂, 24.5 % CaO, 24.5 % Na₂O and 6.0 % P₂O₅) granules obtained from a commercial source (Mo-Sci Corp., Rolla, MO) were used for the study.

Method development

Numerical. The simulated microgravity environment of the RWV is completely different from conditions previously used for BG testing. These unique dynamic conditions are characterized by a lack of sedimentation of the particles immersed in the medium, viscous flow (low Reynolds number flow), low turbulence, low shear stresses at the particle interface, and a dual mass transport mechanism – diffusion and convection. Since the RWV is completely filled with an air bubble-free medium, the fluid motion is not affected by wall boundary layers.¹¹

It is known that a small microcarrier particle normally remains suspended in the RWV, as centrifugation and the fluid drag force balance sedimentation. For conditions used in this study, the resulting time-averaged microgravity experienced by the particle is evaluated to be ~ 0.01 g.

Simulated microgravity conditions for high-density (2.5 g/cm³) BG particles could be effectively maintained by using proper experimental parameters. The choice of the parametric conditions was based on the numerical simulation of the motion of a microcarrier particle in the RWV. In particular, the motion of a single spherical BG granule in the HARV was calculated using the methods described by Gao et al.¹¹ After considerable numerical experimentation, parameters for computation were set as follows: granule size range: 40-70 μm, rotating speed: 10 rpm, the liquid density and viscosity: $\rho_1 = 1.0 \text{ g/cm}^3$ and $\mu = 0.01$

$g/(\text{cm} \cdot \text{s})$. The trajectory of the particle was numerically evaluated in both an inertial and a rotational frame. The trajectories of a 40 μm or a 70 μm particle in the inertial frame of reference (Figure 1 a, b) display a very slow movement of the particle of either size away from the initial location. This suggests that the BG particles of this size range will move along with the bulk fluid for an extended duration of time.

Experimental

Simulated microgravity conditions. A High Aspect Ratio Vessel (HARV-50 ml, Synthecon, Houston, TX) which rotates about a horizontal axis was used to simulate the microgravity environment.

On the basis of the computational study, we selected a BG granule size 40 - 70 μm and a rotational speed of 10 rpm to maintain simulated microgravity conditions in the HARV. Considering that the choice of the experimental parameters was based on the numerical analysis of the motion of a single spherical BG particle, further experimental validation of simulated microgravity conditions for the actual testing conditions involving many particles of an irregular shape was required. This was conducted as follows.

A trial experiment to observe the trajectory of the granule movement in a HARV environment was performed using the chosen experimental parameters. The BG particles were loaded in the HARV at a 1mg/ml mass-to-solution-volume ratio. The calculated quantity and the total surface area of particles loaded at this ratio were 350×10^3 and 24 cm^2 , respectively. The trial experiment was conducted using Au sputter coated BG granules. The Au surface film rendered the particles visible in solution. The observations confirmed that most of the particles, loaded in the HARV using the parameters selected, circled around the central region of the rotating chamber during the 24 hours of observation time. There was no evidence of clustering or aggregate formation during particle rotation in the HARV. Thus, the trial experiment confirmed that, for the selected experimental parameters, simulated microgravity conditions were in fact maintained in the HARV within the time frame of the experiment.

Control unit gravity conditions. BG behavior under simulated microgravity conditions of the HARV was compared with that at normal gravity. Control tests at normal gravity were conducted at both static and dynamic conditions. Well-controlled flow conditions were used for both control experiments. In comparison to the simulated microgravity conditions of the HARV, the static conditions provided a stagnant, surface shear and convection-free environment. Initially, we planned to conduct both control experiments in the HARV. However, a trial experiment showed that the BG behavior under static conditions of the HARV was similar to that in static vials. In view of this, the rest of control experiments at static conditions were conducted in static vials.

In order to determine the effect of gravity on the BG behavior in a parametric study, the control dynamic (motion) test at unit gravity was also conducted in a well-controlled environment of the HARV. The NASA-designed HARV normally rotates around a horizontal axis to create simulated microgravity conditions (HARV, micro-g). A modified HARV, which rotates around a vertical axis, was used for dynamic control studies at normal gravity (HARV, unit-g). In order to perform this experiment, a support system, needed to keep the HARV base in a vertical position, was designed and built. The rest of testing parameters, such as rotational speed (10 rpm), particle size, the ratios of particle mass and surface area to solution volume, were kept identical to before. Thus, the only difference between the two dynamic tests in the HARV was the presence or the absence of simulated microgravity.

Immersion experiments. All immersion experiments, either under simulated microgravity conditions or control unit gravity conditions, were conducted using previously developed test protocols.⁸

Immersion to study BG physico-chemical reactions. These immersion experiments were conducted using three types of simulated physiological solutions: ion- and serum-free tris buffered solution (solution denominated T, pH 7.4 at 37°C); this solution complemented with electrolytes typical for plasma (TE), and TE with 10% serum (TES). The BG particles were immersed at a 1 mg/ml mass-to-solution volume ratio. The corresponding surface area-to-solution volume ratio was 0.48 cm⁻¹. All solutions were pre-warmed to 37°C prior to immersion. The duration of immersion in T was 1, 3, 6, 10 and 24 hours. The immersion time was extended to 48 hours in TE and TES. At least two samples were used for each of the immersion periods.

Post-immersion, the solutions were analyzed for changes in the Si, Ca and P-concentrations using atomic absorption spectrophotometry (5100 PC, Perkin Elmer, Norwalk, CT) or colorimetry (UV-visible spectrophotometer, Ultraspec Plus, Pharmacia Biotech, Piscataway, NJ). The change in surface character of the BG particles was determined using diffuse reflectance Fourier transform infrared spectroscopy (FTIR) (5DXC, Nicolet, Madison, WI).

Immersion to study the adsorption of serum proteins. BG particles used for the protein adsorption study were either unmodified (BG-UN) or modified. When modified, either an amorphous calcium phosphate (BG-ACP) or a carbonated hydroxyapatite (BG-HA) was formed at the BG surface. For adsorption of serum proteins, BG particles were immersed in a typical tissue culture medium (Minimum Essential Medium, MEM) complemented with 10% newborn bovine serum for 2 hours. Proteins were extracted by treating BG particles with 1% Triton-X100 solution three times. The concentration of extracted proteins was measured using Bio-Rad detergent compatible (DC) assay and colorimetry at 750 nm. The amount of extracted proteins was normalized for particle weight.

Results and discussion

The effect of simulated microgravity on BG reactions in solution. Changes in the [Ca], [P] and [Si] as a function of BG immersion time and immersion conditions (simulated microgravity conditions of the HARV versus control conditions), are illustrated in Figure 2. The data show that the BG reactions in all solutions tested (T, TE, and TES) include leaching of all ions, followed by P-uptake and a slowing down of the Si- and Ca-release rate. Although the sequence of the reactions, typical for BG, was observed under both control and simulated microgravity conditions, the kinetics of the reactions and the reaction products were noticeably different.

Table 1. Rates of ion release under simulated microgravity (micro-g) conditions of the HARV versus control unit gravity (unit-g) conditions

Ion	Release rate, $\mu\text{g}/\text{mg h}^{-1}$					
	T		TE		TES	
	micro-g	unit-g	micro-g	unit-g	micro-g	unit-g
SI	50.0	20.0	50.0	25.0	50.0	10.0
CA	80.0	25.0	55.0	5.0	60.0	4.8
P	12.0	2.0	7.0	0.7	ND	ND

Note: Release rates were determined at the linear portion of the ion release plots
nd – not determined

Table 2. Reaction products: [Ca]x[P] ionic product and total amount of P uptake ($M \cdot 10^{-3}$) Simulated microgravity (micro-g) conditions versus control unit gravity (unit-g) conditions.

Solution	[Ca] x [P]		P-uptake	
	micro-g	unit-g	micro-g	unit-g
T	1.04	0.11	0.35	0.03
TE	4.57	2.6	0.87	0.52

Note: The [Ca] and [P] values in the [Ca]x[P] product were determined as [Ca]max and [P] max released prior to P-uptake; P-uptake was determined as a difference between [P]max and [P]min

We determined the effect of simulated microgravity on the following parameters of BG reactions in solutions tested: rates of Si-, Ca- and P-ion release; the amount of ion release; the [Ca]x[P] ionic product, and the total amount of subsequent P-uptake. This comparative analysis revealed a remarkable increase in the rates of ion release in all solutions tested in the HARV (Tab. 1). For instance, in the case of immersion in T, the rates of Si-, Ca-, and P-release in the HARV were 2.5, 3.2 and 6 times greater than those under control conditions. Accordingly, the amounts of Si-, Ca-, and P-release also increased. The ion release in T, expressed as mass % of the original Si-, Ca-, and P-content in BG, was 30%, 60%, and 46.6% in the HARV in comparison to 11.1, 18.3 and 12.9% under control conditions.

The enhancement of BG dissolution under simulated microgravity conditions affected the subsequent BG reactions, which include solution saturation with Si-, Ca- and P-ions followed by BG surface stabilization and formation and growth of Ca-P phases. As shown in Figure 2, in comparison to control conditions, the surface stabilization (indicated by slowing down in the Si- and Ca-release) occurred much faster under simulated microgravity. The enhanced dissolution also resulted in about a 10-fold increase in the [Ca] x [P] ionic product (Tab. 2). This increase was followed by faster and greater P-uptake, which is indicative of growth of Ca-P phases at the BG surface. The time to P-uptake was reduced (1 vs. 6 hours in the HARV and control conditions respectively). There was about a 10-fold increase in the total amount of P-uptake (Tab. 2).

Therefore, the chemical analysis suggests that simulated microgravity environment of the HARV enhanced the BG dissolution and subsequent formation of Ca-P phases at the BG surface.

The effect of simulated microgravity on BG surface modification. FTIR spectra of the BG particles before and after immersion in serum-free TE under simulated microgravity (HARV micro-g) and control conditions are shown in Figures 3. Both control unit gravity tests, static and dynamic (HARV unit-g), were used for these experiments. The spectra after immersion show the presence of broad P-O bands, which were absent before immersion. These bands are characteristic for amorphous calcium phosphate (ACP). Both control conditions showed weak P-O bands. In contrast, simulated microgravity conditions showed unusually large intensities of the P-O bands. Similar effect of the simulated microgravity conditions was observed on immersion in serum-containing TES. The large increase in the intensities of the ACP bands suggests a significant increase in the amount of the Ca-P phase at the BG surface under simulated microgravity conditions.

Thus, both chemical and FTIR analyses suggest that the formation of bioactive Ca-P surface was enhanced under simulated microgravity conditions of the HARV.

The effect of simulated microgravity on adsorption of serum proteins. Figures 5 a and 5 b show the amount of adsorbed serum proteins. Figure 5 a demonstrates the effect of BG surface modification on protein adsorption in unit gravity; and Figure 5 b shows the effect of simulated microgravity. It was found that the amount of proteins adsorbed onto unmodified BG-UN particles in unit gravity was 10 times larger than that reported for porous BG discs.³ In comparison to BG-UN, the amount of proteins adsorbed on modified BG-ACP and BG-cAp particles increased more than two and three times, respectively. The effects of ACP and c-HA phases on protein adsorption are in agreement with previous reports.³ The adsorbed amount became significantly greater under simulated microgravity conditions: the amount adsorbed onto both unmodified and modified BG doubled (a 100% increase).

The study demonstrated the major effects of a large surface (the mean surface area of 40-70 μm particles is 0.48 cm^2/g), surface modification, and simulated microgravity conditions on the adsorption of serum proteins on the BG microcarriers.

Numerical study

Simultaneous to the experimental study, numerical models were developed to evaluate the mass transport of chemical species to and from the BG surface under static and HARV conditions.² It may be noted that under static control conditions, diffusion is the mass transfer mechanism, while under simulated microgravity conditions of the HARV, mass transfer involved both diffusion and convection. As shown in Figure 4, the numerical results for time-dependent changes in the ion concentrations showed an excellent agreement with the experimental data at both static and HARV conditions.

The numerical study suggests that the unique dynamic conditions - lack of sedimentation, very low shear stress (1.0 – 1.5 dyne/cm^2), and the dual mechanism of the mass transport involving diffusion and convection – are likely to be at the basis of the enhanced BG surface reactions under the simulated microgravity conditions of the NASA-designed rotating wall vessel bioreactors.

Conclusions

- This pioneering study revealed the major effect of the unique dynamic simulated microgravity conditions of NASA RWV bioreactors on the surface reactions of bioactive glass (BG) microcarriers.
- These simulated microgravity conditions remarkably enhanced BG reactions in solutions; this enhancement was followed by the enhanced formation of bone-like minerals at the BG surface.
- The study also revealed a remarkable effect of a large surface area, surface modification and simulated microgravity on the adsorption of serum proteins on the BG surface.
- The study demonstrated that the surface reactions critical for BG to function as a substrate for 3-D bone tissue engineering are enhanced in the RWV bioreactors simulating microgravity conditions.
- These findings suggest that the unique, well-controlled, dynamic environment of the RWV bioreactors could be successfully used for engineering biomimetic properties of biomaterials.

Acknowledgement: NASA NRA grant NAG 8-1483

References

1. Radin S, Ducheyne P, Ayyaswamy PS, Gao H. 2001. *Biotech & Bioeng* 75(3):369-378
2. Gao H, Ayyaswamy PS, Ducheyne P, Radin S. 2001. *Biotech & Bioeng* 75(3):379-385
3. Vico L, Alexandre C. 1992. *J Bone Miner Res Suppl* 2:S445-S447
4. Hughes-Fulford M. 1991. *Experim Gerontology* 26(2-3):247-256.
5. Jessup JM, Goodwin TJ, Spaulding G. 1993. *Cell Biochem* 51:290-300.
6. El-Ghannam A, Ducheyne P, Shapiro IM. 1997. *J Biomed Mater Res* 36:167-180.
7. Hench LL. 1991. *J Am Cer Soc* 74(7):1487-1510
8. Radin S, Ducheyne P, Rothman B, Conti A. 1997 *J Biomed Mater Res* 37:363-375
9. Radin S, Ducheyne P, Falaize S, Hammond A. 2000. *J Biomed Mater Res* 49:264-272
10. Garcia AJ, Ducheyne P, Boettiger D. 1998. *J Biomed Mater Res* 40:48-56
11. Gao H, Ayyaswamy P, Ducheyne P. 1997. *Microgravity Sci Technol X/3*:154-165

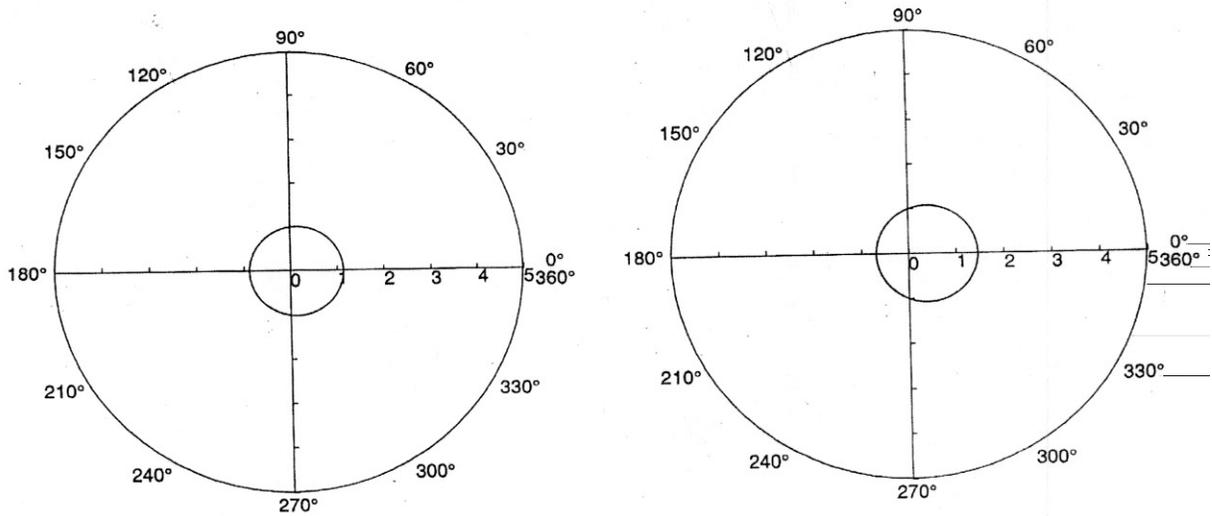


Figure 1. Trajectory of a round solid particle in the HARV in the inertial frame of reference at the selected experimental parameters: (a) $r = 20 \mu\text{m}$, (b) $r = 35 \mu\text{m}$; $\rho_p = 2.5 \text{ g/cm}^3$, $\omega = 10 \text{ rpm}$.

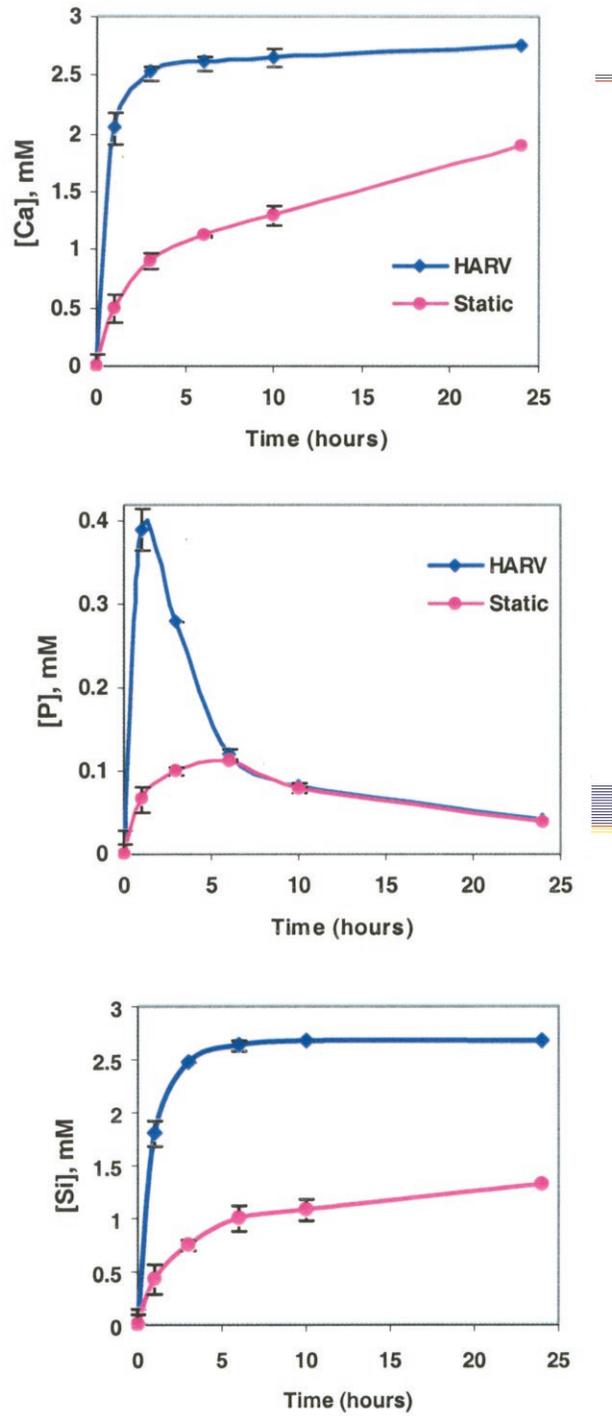
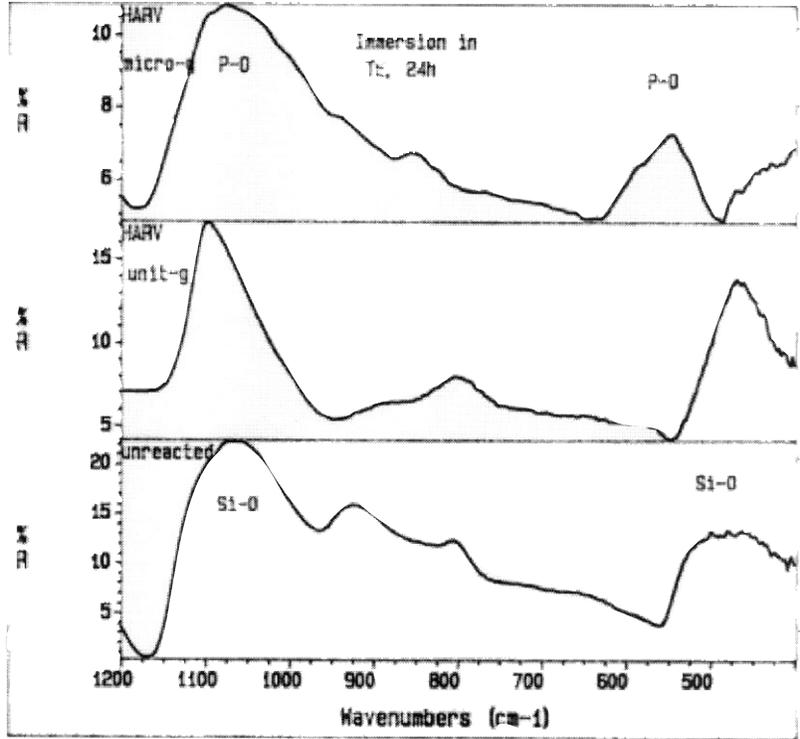


Figure 2. Changes in the [Ca], [P], and [Si] as a function of immersion time in ion- and serum-free T under simulated microgravity conditions of the HARV and control unit gravity conditions.



Figures 3 a, b. FTIR spectra of BG before and after immersion in serum-free TE under simulated microgravity (micro-g) and control unit gravity (unit-g) conditions. FTIR spectra of BG after immersion under both control conditions, static and dynamic, were similar.

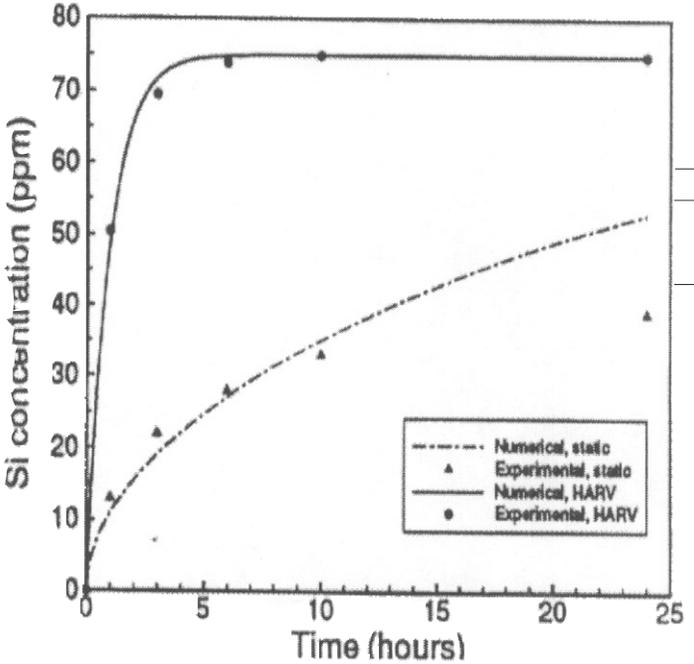


Figure 4. Numerical and experimental data for time-dependent Si-release in T under simulated microgravity conditions of the HARV and control (static) conditions.

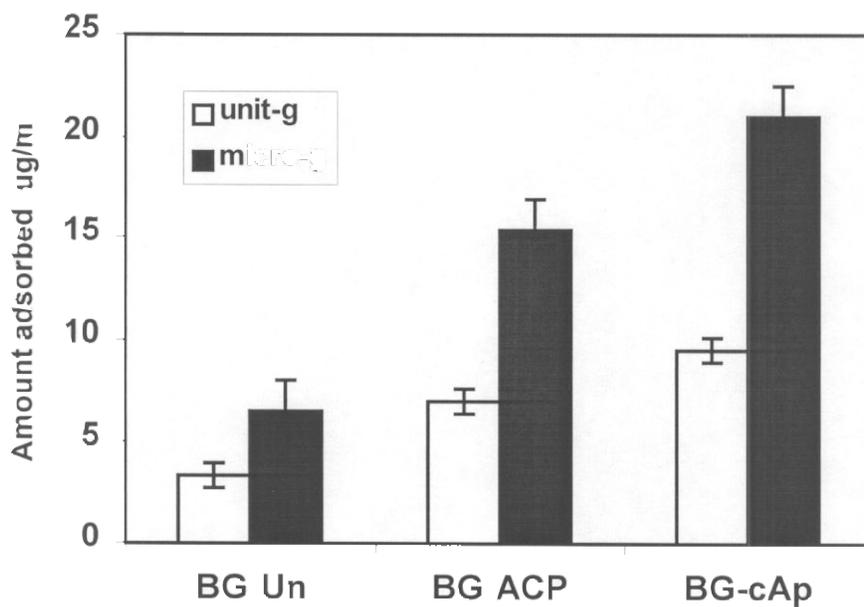
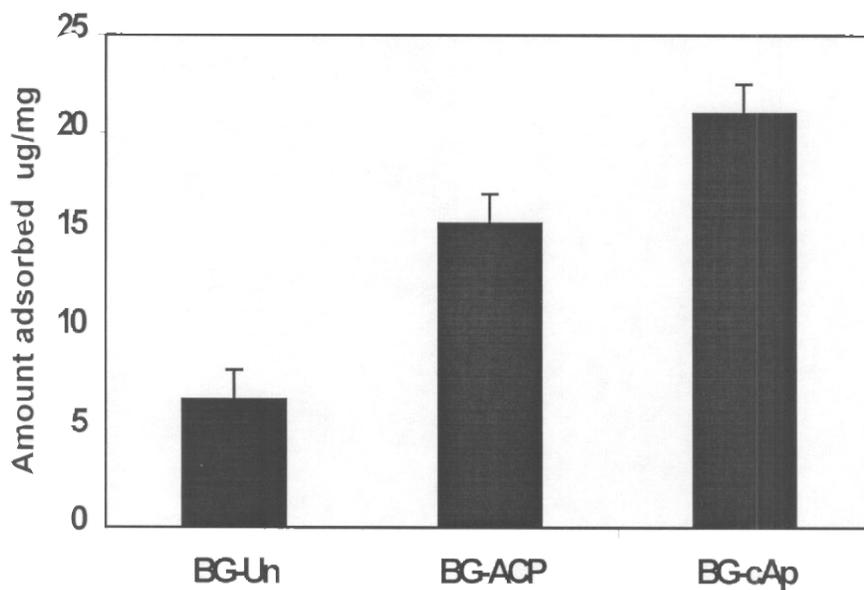


Figure 5 a, b. The amount of adsorbed serum proteins: (a) the effect of BG surface modification (unmodified BG-UN, modified to form amorphous calcium phosphate (BG-ACP), and modified to form carbonate apatite (BG-cAp)); (b) the effect of surface modification and simulated microgravity.